Kidney International, Vol. 46 (1994), pp. 297–309

EDITORIAL REVIEW

Digoxin-like inhibitors of active sodium transport and blood pressure: The current status

More than 30 years ago it was suggested that sodium homeostasis was, in part, attributable to a hormone which could increase sodium excretion by reducing sodium reabsorption in the nephron. Early experiments [1] showed that acute volume expansion in the dog was associated with a blood borne substance which was not aldosterone but which nevertheless increased sodium excretion in a second, cross-circulated, animal. There followed an extensive literature demonstrating that volume expansion was associated with a substance in plasma or urine which decreased the activity of the sodium transporting enzyme Na/K-ATPase [2], and it was assumed that the natriuresis of volume expansion was therefore the result of tubular rejection of sodium through inhibition of the sodium pump by one natriuretic substance. De Bold's discovery [3] of the atrial natriuretic factor and the subsequent observation that this substance does not inhibit active sodium transport [4] showed that at least two substances had been involved in the previous investigation, and led to a focus on the identification of a Na/K-ATPase inhibitor. Many years later we are still in a position of ignorance concerning the role of this endogenous inhibitor of active sodium transport. The purpose of this review is not to detail every advance or reversal in the attempts at identification of this substance, but to attempt to explain why progress has been slow, and to highlight the recent and most interesting observations which have led to endogenous sodium transport inhibitors being the subject of renewed attention.

There are some obvious reasons for the intermittent periods of re-interest in this field, the most overt being the proposal, in the late seventies and early eighties, that sodium transport inhibitors could be involved in the etiology of essential hypertension (EHT). Some of the earliest evidence had suggested a role for a hypertensinogenic substance in the pathogenesis of hypertension in salt-sensitive rats, probably a low molecular weight stable compound which could be transferred to other animals by parabiosis, leading to an increase in their blood pressure [5]. In 1976 Haddy and Overbeck [6], finding that the sodium pump was inhibited in the vasculature of animal models of hypertension, suggested that the circulating pressor compound might be an inhibitor of the sodium pump. Blaustein [7] then proposed that inhibition of the sodium pump might directly increase vascular smooth muscle tone leading to an increase in peripheral vascular resistance and, ultimately, de Wardener and MacGregor [8] published a hypothesis in this journal that argued strongly for an important role for such a factor in the mechanism of EHT. They proposed that a hereditary renal defect in the excretion of sodium in EHT results in the elaboration of a Na/K-ATPase inhibitor with both natriuretic and pressor activity. The pressor action would further encourage natriuresis. This attempt to explain the mechanism of hypertension provoked extensive experimentation, some of which suggested that the plasma of patients with EHT reduces active sodium transport in cells from normotensive individuals [9–13] and of these studies a number showed a quantitative relationship between the amount of serum Na/K-ATPase inhibiting activity and blood pressure in patients with EHT [10, 11, 13].

In parallel with these investigations, and subsequently, a major effort has been directed toward the identification of the contributory substance, or, substances. Recent reviewers have made heroic attempts to summarize this contradictory literature and to put it in perspective [2, 14–16] and the most confusing aspects require some explanation. Firstly, the major research effort has been towards the identification of a sodium transport inhibitor and the original hypothesis that it should also be natriuretic has largely been ignored in that few active fractions have been tested for natriuretic activity. Furthermore, inhibition of the sodium pump in normal subjects given digoxin does not result in a natriuresis [17] although cardiac glycosides will cause a natriuresis when infused selectively into the renal artery at very high concentrations [18]. A dissociation between inhibition by endogenous inhibitory fractions of the sodium pump and natriuresis has clearly been shown in two studies in which fractions of urine from uremic patients have been tested for sodium pump inhibition and natriuresis in conscious rats [19, 20]. One cannot make any assumptions therefore that the many characterized or partially characterized endogenous sodium pump inhibitors will make any contribution to sodium homeostasis.

Another area of obvious confusion is that the search has not been confined to the plasma of volume expanded humans, or indeed patients with EHT. Sodium transport inhibitors (which have included peptides, steroids and lipids) have been isolated from innumerable sources, including most organs studied, and from the serum and urine of normovolemic animals and humans [2, 14, 15]. Circulating sodium transport inhibitors have also been reported in states of pathological volume expansion including uremia [21–24], congestive heart failure [25], following myocardial infarction [26] and in patients with liver disease [27]. A further problem lies in the methods used in characterization. Simply stated, insufficient purification has led to the production of crude "active fractions" identified by a host of different assays, each with its own inbuilt potential for non-specificity. This has resulted in deplorable confusion [14, 15]. Fortunately, this is now widely appreciated, although some laboratories persist in using inappropriate techniques. Lastly, two further areas which expand the original hypothesis have recently yielded interesting data. These

Received for publication April 8, 1993
and in revised form August 2, 1993
Accepted for publication August 2, 1993

include developments concerning the relationship between sodium transport and vascular tone, and the suggestion that the brain may play a fundamental role in the effector pathways of sodium transport inhibitors or indeed may be a site for their synthesis.

The assays used: How have they contributed to the confusion?

In the search for an endogenous inhibitor, the measurement of Na/K-ATPase activity might appear straightforward. The isolated enzyme is most commonly prepared from dog or rabbit kidney and is commercially available. However, in view of the heterogeneous distribution of the three α isoforms of the enzyme in different tissues and their variable affinity for cardiac glycosides [28], the source of enzyme may be more critical than originally thought. Indeed, the α-2 isoform, which predominates in canine aorta, is particularly sensitive to ouabain [29]. In view of the interest in the role of endogenous sodium transport inhibitors and peripheral vascular tone in hypertension, there would therefore be a strong case for using a vascular smooth muscle preparation of the enzyme in preference to the enzyme from the kidney which, at least in the rat, contains only the α-1 isoform, known to be resistant to cardiac glycosides. The methods of measurement of activity of isolated Na/K-ATPase are well proven, although, as detailed below, they may be of limited value for detection of endogenous material. Alternative methods are available including: the measurement of sodium efflux [9, 12, 13] or ^{86}Rb (equivalent to potassium) influx in cells [30]; the displacement of radiolabeled cardiac glycosides from their specific receptor site on Na/K-ATPase [31]; the short circuit current across toad bladder [32]; and cross reactivity with antidigoxin antibodies [33]. This heterogeneity alone is complexing, but is further compounded by problems specific to the individual assays.

Na/K-ATPase enzyme activity

Na/K-ATPase is a highly complex molecule embedded in the cell membrane. The enzyme requires many ligands and substrates for cycling to occur, and for the binding of cardiac glycosides. Changes in the availability of each of these influence enzyme activity and can give rise to non-specific inhibition. As it is not embedded within a cell membrane and lacks the protein buffering component of the cell membrane [34], the isolated enzyme is highly susceptible to this form of inhibition and is liable to be inhibited from both external and cytosolic surfaces [24, 35, 36]. For example, in our laboratory we have found only two adjacent fractions of human neonatal serum (fractionated by high pressure liquid chromatography) to inhibit leukocyte sodium transport, yet nearly all fractions inhibit isolated Na/K-ATPase. The most commonly used assay of isolated Na/K-ATPase activity [11] in which ATP generation is linked to the oxidation of NADH involves two other enzymes, lactate dehydrogenase and pyruvate dehydrogenase; inhibition of either of these leads to a false suggestion of Na/K-ATPase inhibition. The assay is normally carried out with saturating concentrations of ATP which *in vivo* might not be the case in many cells. Alternatively, problems could arise from substantial ATP depletion due to greatly increased Na/K-ATPase activity or excess pyruvate which reduces NADH and depletes ATP [23]. Many ions, other than sodium and potassium, which can inadvertently be concentrated by fractionation techniques, can affect isolated Na/K-ATPase activity including protons, magnesium and calcium [37], vanadate [38], and zinc [39]. Ascorbic acid also inhibits the isolated enzyme [40] by reducing a group within the ATP binding site, but as this site is on the inner side of the membrane, this effect is not apparent in cellular preparations.

Cellular sodium and rubidium transport

Many studies have employed intact cells, particularly erythrocytes [9, 41, 42] and leukocytes [9, 11, 13] and more occasionally, renal tubular cells [43], to test for inhibition of the sodium pump. Intracellular sodium, or outward fluxes of sodium or inward transport of ^{86}Rb (as an index of potassium transport) are measured and the cardiac glycoside sensitive component of the flux taken to represent activity of the sodium pump. These assays are much more specific than those using isolated Na/K-ATPase, but if any compound affects oxidative phosphorylation and therefore cellular ATP, or if it has a detergent effect on the cell membrane, the sodium pump will be inhibited although digitalis receptor occupation has not occurred. Lipids, particularly negatively charged phospholipids, are an absolute requirement for ATPase activity [44] and alterations in the lipid environment by uptake from the extracellular medium may inhibit or stimulate activity [44–46]. Free fatty acids directly inhibit cellular sodium transport [47] and isolated Na/K-ATPase [48] but, as detailed below, inhibition of Na/K-ATPase by lipid containing fractions is more likely to result from a "detergent" effect and is unlikely to represent the presence of a specific ligand. Also, in view of the different distribution of isoenzymes of Na/K-ATPase, the choice of cell may be all important in determining the response to circulating inhibitors.

Displacement of radiolabeled cardiac glycosides

This assay might be of particular value in the detection of an endogenous cardiac glycoside, since an inhibitor competitively displacing ouabain or digoxin from the binding site on Na/K-ATPase could theoretically suggest the presence of a natural ligand. Displacement of ^{3}H-ouabain has been used in the search for an endogenous ligand in the serum of hypertensive subjects [31], neonates [30], normal human serum [49], in fractions of human urine [50, 51], rat brain [52], and bovine adrenal glands [53]. Disadvantages include detergent activity of impure fractions and the influence of external potassium concentration (which has usually been ignored in studies using whole serum) both of which influence ligand binding. Binding studies involve extensive washing of the enzyme/cells when the inhibitor may be lost, and allowance has also to be made for a degree of non-specific binding which occurs when high concentrations of ^{3}H-ouabain are exposed to Na/K-ATPase.

Cytochemical assay

Some of the earlier work involved in the detection of endogenous inhibitors of the sodium pump was based on a cytochemical assay for inhibition of Na/K-ATPase in sliced guinea pig kidney and it was shown that the fractions which inhibit Na/K-ATPase by this method also stimulated glucose-6-phosphate dehydrogenase (G6PD) [54]. Subsequent work, however, showed that the fractions which cytochemically could be shown to inhibit Na/K-ATPase and stimulate G6PD *in situ* neither inhibited isolated Na/K-ATPase nor demonstrated binding to a digoxin antibody [19].

Digoxin radioimmunoassay

Most commercially available digoxin antibodies demonstrate high affinity for digoxin and will not bind to cardiac glycosides other than those which are closely related in structure. The antibodies are generally directed against a specific portion of the steroid moiety of the digoxin molecule (a discrete area associated with position 12 on the steroid backbone) [55]. Many groups have assumed that an endogenous cardiac glycoside should bind to digoxin antibodies and "endogenous digoxin-like immunoreactivity" (EDLI) has frequently been used to detect endogenous sodium transport inhibitors. Plasma EDLI was first described by Besch et al [56] following the observation of unexpectedly high laboratory values for serum digoxin in premature infants receiving digoxin therapy. EDLI attracted public attention when, in Toronto in 1981, very high levels of serum "digoxin" were found *post mortem* in a number of babies not receiving digoxin therapy, and a nurse was accused of murder. Fortunately for the defendant it was eventually recognized that all neonatal serum gave false positive values for digoxin. EDLI in neonatal serum is often much greater than serum digoxin in a fully digitalized subject, but is highly variable depending on the assay used [57]. The serum of the neonate does inhibit active sodium transport in a biological assay [30, 58–62] but a relationship between biological activity and EDLI is found by some [30, 61] and not others [59, 62].

Pregnant women have raised EDLI which increases further in pregnant women with hypertension [63–65]. Again no relationship between EDLI in pregnancy and activity in a biological assay has been found despite evidence of a sodium pump inhibitor in the serum of women with mild gestational hypertension [65]. Some or all of the EDLI in the neonate and pregnant women is likely to result from non-specific cross reactivity of the antibody with the high concentrations of steroids known to be elevated in pregnancy [66, 67], yet not all reports agree that steroids play a role [30, 62, 63].

Further evidence for a dissociation between EDLI and biological activity has been reported in a dialysis dependent patient [22], in patients with acute myocardial infarction [26], in EHT [11, 12], in normal human plasma [49, 68], in human urine [50], in plasma of saline loaded rats [69], and in *in vitro* studies of a number of peptides and steroids [70].

The commercially available Fab fragment of the digoxin antibody binds cardiac glycosides other than digoxin, and there is some evidence largely from investigations of endogenous inhibitors in cord blood that it may have affinity for an endogenous ligand of Na/K-ATPase. Montali, Balzan and Ghione [71] have used the Fab fragment to partially purify an inhibitor of erythrocyte ^{86}Rb uptake from neonatal serum [71]. The antibody fragments were covalently bound to Sepharose and incubated with serum. Elution with methanol produced a fraction rich in inhibitor. The Fab fragment also reversed the inhibition of the sodium pump achieved by incubation of normal erythrocytes with serum from cord blood [72] and has been found to inhibit the pressor activity of a centrally administered hypothalamic ouabain-like substance [73] (see below). In addition, Goodlin [74] has found that infusion of the Fab fragment into one woman with pre-eclampsia caused a profound fall in the blood pressure, but this has not been repeated.

The non-specific binding of digoxin antibodies to endogenous compounds which do not possess cardiac glycoside-like characteristics has probably contributed more to the confusion in this field than any other single factor [14]. Indeed in his recent review Goto et al have deliberately omitted all reference to data achieved using this assay [15].

Summary

In view of these pitfalls, and in the absence of any ideal assay, there is now general agreement that any candidate compound for an endogenous inhibitor of sodium transport should be tried and tested by a combination of at least two assays, one of them cellular sodium or rubidium transport [15]. Fortunately, many of the studies of the last two or three years have adopted this approach. This, together, with painstaking attempts at complete purification has at last begun yield to a degree of conformity in the characteristics of a number of compounds, and in some to positive identification.

Identified endogenous inhibitors of the sodium pump

Lipids

In the 1980s several investigators identified fatty acids as circulating sodium pump inhibitors. Linoleic acid was shown to be associated with fractions which inhibited Na/K-ATPase from hog plasma [75] and boiled human plasma [76], and oleic acid with fractions from hog [75], bovine [77] and human plasma [76]. There was some physiological corroboration of this as levels of free fatty acids increase after saline infusion [75, 78]. More recently, Lichtstein et al [79] have extracted to purity the novel unstable hydroxy unsaturated fatty acid derivative 11,13-dihydroxy-14-octodecanoic acid from bovine serum which inhibits ^{3}H-ouabain binding and microsomal Na/K-ATPase. *In vitro* studies clearly show that free fatty acids inhibit cellular sodium transport and isolated Na/K-ATPase [48, 80], but inhibition may well be non-specific [81]. All these Na/K-ATPase assays of "active fractions" or of the lipids themselves have been carried out in the absence of protein and may be misleading since, *in vivo*, free fatty acids are more than 98% protein bound [82] and unlikely to be "free" for binding to Na/K-ATPase. The addition of albumin therefore almost eliminates the inhibition of Na/K-ATPase by free fatty acids and lisophosphatidylcholines [83], and it is considered unlikely that these lipids will act as circulating modulators of the sodium pump. Lisophosphatidyl choline [84], a derivative of lisophosphatidyl choline [85], and a derivative of lisophosphatidyl serine [86] have also been isolated from active fractions of plasma. Again, these are unlikely to represent circulating inhibitors of Na/K-ATPase as the inhibitory activity of lisophosphatidyl choline disappears in the presence of protein [83].

Ouabain

Some recent very detailed studies indicate that the tissues and plasma of animals and humans contain the cardiac glycoside, ouabain.

In 1989, Hamlyn, Harris and Ludens [36] found one discrete peak of biologically active material isolated from human plasma of subjects undergoing routine plasmapheresis which inhibited ^{86}Rb uptake in human red cells, isolated Na/K-ATPase and competed with ^{3}H-ouabain for binding to Na/K-ATPase. The active peak contained a compound with very high affinity for Na/K-ATPase—much greater than any known cardiac glycoside which stabilized the E_2P form of Na/K-ATPase in a manner

analogous to ouabain. It did not bind to digoxin antibodies and had no inhibitory effect on H^+,K^+-ATPase or sarcoplasmic reticulum Ca-ATPase. Subsequently, the same group [49, 87] introduced two refinements in the preparation including an enzyme-affinity purification stage which increased the final yield. A total of 300 liters of human plasma was processed to yield 31 μg of the pure inhibitor, representing purification on a dry weight basis of 0.6 billion-fold. The isolated substance was considered to be the same compound but purer than that obtained in the earlier study [36], although it did not possess such high affinity for Na/K-ATPase, being similar to that for ouabain. Using fast atom bombardment mass spectrometry [88], a single protonated molecular ion with a molecular weight of 585.2 Da was identified. Together with the elemental composition deduced to be $C_{29}H_{45}O_{12}$, the characteristics of this compound matched those of ouabain (585.291 Da). Linked scan tandem mass spectrometry showed that the spectra of EDLF obtained were found to be identical to those of ouabain. In addition, the endogenous "ouabain" bound with high affinity to specific ouabain antibodies [89]. These antibodies have high specificity for ouabain and low affinity for other endogenous steroids and give "ouabain" concentrations in human plasma of approximately 0.6 to 1.0 nmol/liter. The therapeutic levels of digoxin range from 1 to 3 nmol/liter and as human Na/K-ATPase, at least in the heart, has twice the affinity for ouabain as for digoxin, the circulating concentrations of "ouabain" may be sufficient to exert a physiological effect [90]. This is supported by our *in vitro* studies which have shown that 0.1–10 nmol/liter of ouabain has a significant effect on vascular function in human resistance arteries [91, 92, see below]. These data cannot completely exclude the possibility that the active compound is an isomer of ouabain, nor confirm that the sugar moiety is definitely rhamnose, the glycoside element of ouabain. L-rhamnose is not normally associated with mammals but it can be synthesized *in vitro* from glucose by rabbit skin [93] and the enzymes responsible for its metabolism are present in the adrenal and pituitary glands [94]. The discovery of a cardiac glycoside such as ouabain, which has a *cis* junction in the steroidal ring (CD *cis*), rather than the CD *trans* ring junction, as found in mammalian endogenous steroid hormones, is completely novel, and Hamlyn's discovery would doubtless have been met with more universal acceptance had a CD *trans* steroid been isolated. LaBella and colleagues [95] have proposed that a CD *trans* steroid could fill the role of an endogenous sodium pump inhibitor as some CD *trans* steroids demonstrate very high affinity for the cardiac glycoside binding site, amongst them a number of synthetic progesterone derivatives [95, 96].

One of the criticisms often leveled at the discovery of "ouabain" is that the compound is so unlikely to be synthesized in mammals that it must be derived from the diet [97], and a number of dietary compounds including tea [98] and rat chow [99] do contain sodium pump inhibitors. Hamlyn and Manunta [90] also have some evidence that ouabain is present in meat, fish and shellfish but argue that the "ouabain" isolated from plasma was an endogenous compound as it was found in patients maintained on semisynthetic diets for periods of seven days [49], by which time they calculated that dietary "ouabain" should have been cleared.

Another potential concern lies in the origin of the plasma used in the isolation of "ouabain." In one publication the origin of the plasma is detailed to be saline-loaded individuals [49], and in a second, by Ludens et al, as patients with neuropathies [87]. However, the characterization described by Mathews et al [88] was carried out with material obtained from the study of Ludens et al [87] which on that occasion was described as volume expanded plasma. It would appear that the patients from whom plasma was obtained by plasmapheresis were volume expanded patients with neuropathies, some of whom were normally on treatment (not including cardiac glycosides).

The adrenal cortex would appear to be the source of "ouabain" in the rat [100] as adrenalectomy lowers plasma "ouabain." Consistent with this, cultured human and bovine adrenal cortical cells have been shown to secrete "ouabain" [49]. Elevated levels of plasma "ouabain" have been found in two patients with adrenocortical tumors [90] and in the venous blood of patients with congestive heart failure [25].

Two other groups have evidence for an endogenous compound which could be ouabain. Inagami and Tamura [53] isolated a compound from bovine adrenal which inhibits ^{86}Rb uptake into human erythrocytes, isolates Na/K-ATPase, and displaces ^{3}H-ouabain from erythrocytes and binds to highly specific antibodies to ouabain [15]. Goto et al [101] have isolated two compounds from human urine, one more polar than the other. The polar compound is very similar to ouabain in that it inhibits canine kidney Na/K-ATPase, and ^{86}Rb uptake by human erythrocytes, the dose response curves being almost identical to those of ouabain. This "ouabain" also increases with saline loading [102] and with elevation of dietary salt intake [103].

Although, Hamlyn's group has identified "ouabain," the immediate question that arises is whether "ouabain" is associated with volume expansion and/or hypertension. In a study of congestive heart failure there was a relationship between the cardiac index and "ouabain" titer, but "ouabain" concentration was inversely related to the mean arterial pressure and unrelated to left ventricular filling pressure [25]. An increase in "ouabain" was not associated with the volume expansion of mineralocorticoid administration in humans, but was increased in hypertension associated with hypothyroidism [90]. Earlier studies using a different "ouabain" radioimmunoassay described elevated "ouabain" like activity in the serum of patients with EHT [104], but the immunoreactivity was later found not to be specific to ouabain and ascribed to an unstable lipid [105]. Despite the indication in 1991 [49] that "ouabain" was present in human plasma, there is still no evidence that the concentration of "ouabain" is elevated in EHT.

Digoxin

As mentioned above, Goto et al [101] have isolated two compounds from human urine, one of which appears very similar, if not identical to ouabain. The second, less polar compound has characteristics remarkably similar to digoxin [106], having identical characteristics to digoxin by analysis with high pressure liquid chromatography and thin layer chromatography.

Bufodienolides

Before the identification of "ouabain" in human serum, the only positive identification of steroids structurally related to cardiac glycosides in the animal world was in various tissues of the toad *Bufo marinus* [107] and in nuchodorsal glands of a snake [108]. These compounds, the bufodienolides, have six membered lactone rings and are potent inhibitors of sodium transport. Very recently, bufodienolides have been isolated from the lens of the human eye, obtained from patients with cataracts [109, 110].

These compounds, 19-norbufalin and its derivatives, inhibit ^{3}H-ouabain binding and Na/K-ATPase activity. Lichstein has pointed out that as the synthesis of these steroids from digitalis compounds requires a photo-oxidation step, the bufodienolide may be the product of endogenous "digoxin" or "ouabain" in the lens after exposure to ultraviolet radiation [109].

Endogenous active sodium transport inhibitors and the brain

The discussion above has centered on the characterization of circulating sodium transport inhibitors. There is also some recent evidence that such a substance may be present in the brain and may have a local action.

Indirect evidence

In the earliest experiments [111, 112] an anteroventral third ventricle (AV3V) lesion of the hypothalamus was shown to prevent the usual rise in the plasma sodium pump inhibitory activity which follows saline infusion. Hypothalamic catecholaminergic denervation induced by intraventricular injection of 6-hydroxydopamine or an AV3V lesion prevents hypertension in the salt-loaded reduced renal mass (RRM) and in salt-loaded DOCA rats and also prevents the associated increase in sodium pump inhibitory activity [113, 114]. Inhibition of the sodium pump in these early experiments was estimated by ^{86}Rb uptake into normal rat tail arteries incubated in the plasma of the experimental animal. In addition, the administration of hypertonic saline into the cerebral ventricles of dogs causes a rise in blood pressure together with a reduction in ouabain-sensitive ^{86}Rb uptake of branches of the saphenous veins, and of veins from a control animal incubated in the experimental dog's plasma [115]. The rise in intracellular sodium and fall in potassium in certain skeletal muscles in rats given DOCA + saline also appears to be related to a circulating inhibitor of Na/K-ATPase controlled by the hypothalamus. Katafuchi et al [116] observed that in a slow tonic muscle, the soleus muscle, the changes in intracellular sodium and potassium can be prevented by either denervation of the muscle or by bilateral lesions of the ventromedial nuclei. In contrast, the changes in electrolyte content of a fast twitch muscle, the extensor digitalis longus, are unaffected by denervation but they are prevented by either an AV3V lesion or bilateral lesions of the paraventricular nuclei, which implies a hormonally mediated mechanism. Two studies have suggested that there may be hormonally induced release of a sodium pump inhibitor in the brain. The intracisternal administration of pergolyde, a dopamine receptor agonist, triggers the release of a circulating sodium pump inhibitor as measured by ^{86}Rb uptake [117] as does the injection of atrial natriuretic factor (ANF) into the lateral cerebral ventricles [118].

It is also possible that the brain may be responsible for the pressor effect of a locally produced or circulating sodium pump inhibitor. The increase in arterial pressure and renal sympathetic activity induced by the central administration of hypertonic saline may be prevented by intracerebroventricular pre-injection of digoxin-specific antibody Fab fragments but not by intravenous administration of these antibodies [73, 115, 119]. However, this must be interpreted with caution in the light of the potential lack of specificity. Huang and Leenan have also studied the effect in the spontaneously hypertensive rat (SHR) and the WKY rat of high and low sodium diets upon the rise in blood pressure and increase in renal nervous sympathetic activity which follows intracerebroventricular ouabain infusion [120], a response which appears to be due to the effect of the ouabain on the posterior hypothalamus [121]. In the SHR a high sodium diet diminishes the pressor response and increase in renal sympathetic activity to ouabain which were unaffected in the WKY rat. They proposed that the diminished response in the SHR is due to the high sodium diet having already increased the hypothalamic concentration of the endogenous ouabain-like substance to a greater extent than in the WKY [120].

In support of the suggestion that the brain may synthesize a cardiac glycoside, two digoxin antibodies have been found to bind to the hypothalamus (predominantly to the paraventricular and supraoptic nuclei) of the rat, dog and macaque [122–124]. There is also intense binding in the subfornical organ and the organum vasculorum laminae terminalis. The latter is of particular relevance in view of its close proximity to the site of the AV3V lesion. However, no controls were carried out in which the binding of non-specific IgG was carried out and again the non-specificity of the digoxin antibodies must be considered. Nevertheless, the distribution of binding was similar to an antibody raised against ouabain [125].

Direct evidence from extracts of the hypothalamus

The active substance obtained from the hypothalamus in some of the earlier investigations had properties compatible with its being a peptide [126] and a steroid [127]. In keeping with Goto et al's [128] finding in plasma, Huang et al [73] have some evidence (subject to the *caveat* mentioned above) which suggests that the active substance in the hypothalamus and pituitary may be digoxin. Intracerebroventricular injection of an impure extract with an activity equivalent to approximately 1 mmol/liter ouabain caused an increase in blood pressure and renal sympathetic nerve activity which was prevented, or considerably diminished, by an intracerebroventricular pre-injection of digoxin antibody Fab fragments.

Haupert has obtained a sodium pump inhibitor from bovine hypothalamus, the extraction yielding only 750 pmol of material from 1 kg wet weight of tissue [129, 130]. Activity has been demonstrated with the coupled enzyme assay, inhibition of ouabain binding and changes in short circuit current of the toad urinary bladder. The material is water soluble, has a molecular weight less than 1000, and its biological activity is lost after methylation with diazomethane which suggests the presence of a carboxylic group. The substance has functional resemblance to a cardiac glycoside as it is positively inotropic and inhibits the sodium pump in intact renal epithelial cells. However, Haupert et al believe that it is not ouabain or digoxin as: (a) it does not cross react with monoclonal and polyclonal antibodies formed against digoxin; (b) its effect on short circuit current and its inotropic action are rapidly reversible; (c) unlike ouabain it traverses liposomal membranes (but digoxin does); (d) it inhibits sarcoplasmic Ca ATPase, perhaps because of (c); (e) it does not promote phosphorylation of the active site of Na/K-ATPase; and (f) its ionic requirements for optimal inhibition of Na/K-ATPase activity differs from ouabain. A recent study [131] provides chromatographic and spectroscopic evidence that this hypothalmic substance is an isomer of ouabain. It is presumed that the structural difference between these two substances accounts for their different biological properties.

Ferrandi et al [132] have also extracted a hypothalamic pump

inhibitor from Milan hypertensive (MHS) and normotensive (MNS) rat strains and from the ox. Using the same procedure, they have also obtained an extract from the bovine adrenal. The active substance was identified using the coupled enzyme Na/K-ATPase assay, ^{32}P-ATP hydrolysis by ouabain binding, and with erythrocyte ^{86}Rb uptake. Rat and bovine material have the same functional characteristics. The yield from the MHS hypothalamus was about 50× greater than from the MNS and provides the first demonstration of a quantitative relationship between hypertension and a brain-derived ouabain-like sodium pump inhibitor. Activity was also found in the MHS rat but not in the MNS rat adrenal gland. The active material has similarities to ouabain but in common with Haupert's material, it shows no apparent optical peak of UV absorbance recorded at 214 nm (the wavelength of absorbance of ouabain) possibly because of the small amount of material. The only physicochemical difference to ouabain that Ferrandi et al observed was the partial inactivation with treatment with 6 N HCl at 110°C for two hours, whereas the activity of ouabain was unaffected by this short period of exposure to acid. Ferrandi et al's material has two additional biological differences from ouabain. The first is a striking difference in affinity for different preparations of Na/K-ATPase. The hypothalamic material has similar potency to ouabain when synaptosomal Na/K-ATPase is used, but inhibits rat renal Na/K-ATPase with a potency about 1000× greater than ouabain and may reflect α-1 isoform specificity for the endogenous ligand. The functional significance is that this endogenous compound if present in plasma might be natriuretic, in contrast to ouabain (see earlier). In addition, this peculiarity of the renal α-1 isoform of Na/K-ATPase which makes it more sensitive to the hypothalamic ouabain-like substance than to ouabain, to which it is very insensitive [28], appears to change with age for renal Na/K-ATPase obtained from an adult rat is more sensitive to the hypothalamic substance than is renal Na/K-ATPase from a young rat. The effect of exogenous ouabain, however, is unaffected by the age of the rat from which the renal Na/K-ATPase is obtained.

Using cytochemical techniques we have found a substance in the hypothalamus and in the urine which inhibits Na/K-ATPase activity and stimulates G6PD activity [19, 133]. In the amounts available, however, it cannot be detected by the coupled enzyme assay of Na/K-ATPase activity and does not affect leukocyte sodium pump activity. Though apparently not cardiac glycoside-like, it is interesting nevertheless. The cytochemically detectable substance transiently inhibits sodium pump activity in the proximal tubule of the guinea pig kidney with a maximal effect at four to six minutes and transiently stimulates G6PD activity at two minutes. The selectivity of these assays is such that no known mammalian substance has yet been found which acts in this precise manner. The plasma activity of this substance in normal human [133] and the rat [134] rises with an increase in salt intake and is raised in EHT [54], the SHR [135] the MHS [136] and the salt-loaded reduced renal mass (RRM) hypertensive rat [136]. The greatest concentration of the substance in the normal rat is in the hypothalamus where it rises substantially with a high salt intake [138], but even higher concentrations are found in the hypothalamus of the SHR [135] and of the RRM hypertensive rat. The compound has physicochemical properties which suggest that the active substance is highly reactive and contains a functionality of the quaternary ammonium or immonium type [139]. The bioassayable activity of some compounds of this type with the cytochemical assays mentioned above have revealed a close similarity to the hypothalamic substance. To date the most potent of these is the choline analogue di-methyl methylene immonium ion.

Vascular sodium pump inhibition and possible mechanisms of hypertension

The recent identification of "ouabain" as a naturally occurring mammalian hormone has renewed interest as to how inhibition of sodium transport could lead to an increase of vascular tone in humans. One hypothesis, that the brain may mediate the pressor response to a sodium pump inhibitor, has already been discussed. In this section, evidence for a direct vasoconstrictor effect on the vasculature following the inhibition of sodium transport is reviewed.

To investigate the response to sodium pump inhibition in vascular smooth muscle, most experimental studies have made use of cardiac glycosides, such as digoxin and ouabain, and some derivatives of progesterone, which as mentioned earlier bind specifically to Na/K-ATPase. Preparations of vascular smooth muscle have included conduit arteries, resistance arteries and cultured vascular smooth muscle cells. In general, inhibition of active sodium transport has lead to vasoconstriction. Although a definitive unifying mechanism has remained elusive, ultimately contraction of the actin-myosin apparatus in vascular smooth muscle cells only requires a rise in free intracellular calcium. The relative contributions of the neuroeffector junction, vascular smooth muscle and endothelial cells to the change in vascular tone remain contentious and the differing sensitivities to sodium pump inhibition between species make comparison difficult [42].

The neuroeffector junction

Cardiac glycosides may increase the release and reduce the uptake of norepinephrine by sympathetic nerve endings. The increased release is probably mediated by both calcium-dependent and calcium-independent pathways [140–143]. Reduced reuptake results from elevated intracellular sodium which directly inhibits calcium-independent sodium-dependent pre-synaptic re-entry of norepinephrine into the nerve ending [144, 145]. These mechanisms may play a role in the increase in forearm vascular resistance (measured by venous occlusion plethysmography) which follows the intra-arterial infusion of ouabain (0.7 μg/min/100 ml of tissue) as one group has found it to be sensitive to phentolamine, an α-blocker [146]. However, earlier studies showed no effect of α-blockade [147, 148] and those of Hulthen et al [149] who demonstrated no venoarterial difference in norepinephrine concentrations following intra-arterial ouabain infusion. Drug-induced changes in forearm blood flow, however, may make the interpretation of such studies difficult. In the organ bath, phentolamine inhibits neither digoxin-induced contraction of human conduit arteries and veins [150, 151] nor ouabain-induced contraction of human subcutaneous resistance arteries [91]. These studies have used a wide range of concentrations of cardiac glycosides and are therefore difficult to interpret; however, most of the neurophysiological investigations suggest that these mechanism are important at concentrations of 1 μmol/liter or more which is 1000× higher than those reported for ouabain in human plasma [49].

The vascular smooth muscle cell

Depolarization. The sodium pump is electrogenic and contributes to a varying degree (depending on the cell type) to the resting membrane potential. Theoretically, inhibition of this pump in vascular smooth muscle could lead to sufficient depolarization to promote calcium entry through voltage-sensitive channels which may be blocked by antagonists such as verapamil, nifedipine and diltiazem. Robinson et al [148] showed that ouabain increased forearm vascular resistance but demonstrated that the dilator response to intra-arterial verapamil was similar whether the forearm had been treated with ouabain or not implying that calcium entry through voltage-dependent channels was an unlikely cause of the increased resistance. In contrast, nifedipine relaxed digoxin-induced contraction of isolated human conduit arteries [150, 151] and diltiazem inhibited approximately 50% of ouabain-induced vasoconstriction of isolated human subcutaneous resistance arteries [91]. These apparently conflicting data suggest that either the degree of depolarization resulting from sodium pump inhibition may vary between vascular beds, or that the voltage thresholds of calcium channels in vascular smooth muscle differ. Inhibition of the sodium pump leading to depolarization may account for the augmented pressor responsiveness to norepinephrine and angiotensin observed in normal subjects who have received digoxin [152]. Similarly, ouabain potentiates the tone of submaximally precontracted human resistance arteries and rat mesenteric resistance arteries which Aalkjaer and Mulvany have suggested is the consequence of further depolarization [153, 154]. In some vascular smooth muscle preparations, therefore, calcium entry through voltage-gated channels cannot fully explain the increase in tension resulting from inhibition of the sodium pump. Other mechanisms need to be considered.

Na/Ca exchange. The most elegant and arguably most contentious theory to explain tension development following inhibition of the sodium pump is dependent upon the consequent increase in intracellular sodium and the subsequent reduction in the sodium gradient across the vascular smooth muscle cell membrane. This has attracted interest in the light of the elevation of intracellular sodium which has been observed in patients with EHT [155] and pregnancy-induced hypertension [156, 157].

Early observations in cardiac, neural and vascular smooth muscle tissue identified the partial dependence of calcium flux on the transplasmalemmal sodium gradient [158–160]. It was suggested that this was performed by a discrete mechanism, Na/Ca exchange, which in the heart has been identified with a unique carrier protein [161]. The physiological role of this transporter, shared with Ca-ATPase, is to maintain low levels of free cytoplasmic calcium in the vascular smooth muscle cell. It is proposed that reduction of the transplasmalemmal sodium gradient which results from sodium pump inhibition may lead to a rise in calcium entry not only through depolarization but also from the reduction of sodium-dependent calcium efflux by Na/Ca exchange [162]. In support of this, incubation of rat aortic rings in ouabain or low sodium (to reduce the transplasmalemmal sodium gradient) increases the caffeine- and norepinephrine-sensitive calcium stores sequestered in the sarcoplasmic reticulum [163]. In the continuing presence of sodium pump inhibition, saturation of this reservoir leads to raised free intracellular calcium concentration and stimulation of the actin-myosin contractile apparatus, even in the presence of calcium channel blockade by diltiazem or nitrendipine [163]. Blaustein postulated that a circulating sodium pump inhibitor would similarly reduce the transplasmalemmal sodium gradient which has been observed in hypertension [155–157] and therefore increase intracellular calcium leading to a rise in tone [7]. This novel hypothesis provided an exciting link between sodium metabolism and hypertension. Further studies have since suggested that the influx of calcium arising from reduction of the normal transplasmalemmal sodium gradient may depend on the initial concentration of intracellular calcium [163, 164]. This makes Blaustein's theory even more appealing because physiological tone of resistance arteries *in vivo* results from elevated free cytoplasmic calcium concentration in the vascular smooth muscle cells.

The acid test of Blaustein's hypothesis must be its applicability to human tissue. Using human umbilical arteries, Sato and Aoki [165] found that ouabain at concentrations from 300 nmol/liter and above evoked a dose-dependent biphasic response, the early contraction being sensitive to calcium antagonists and the late phase being dependent on external calcium and blockable by amiloride. The effect of amiloride was attributable to inhibition of Na/Ca exchange, but amiloride is non-specific as it also inhibits Na/H exchange and voltage-dependent calcium channels [166]. Sato and Aoki also found that incubation in low sodium produced a concentration-dependent, calcium-dependent and verapamil-independent contraction, highly suggestive of the presence of Na/Ca exchange [165]. Dependence of ouabain-induced contraction on external calcium and Na/Ca exchange has also been suggested in human placental arteries and veins, although again amiloride was considered to be a specific inhibitor of Na/Ca exchange [167, 168]. In contrast, digoxin-induced vasoconstriction of human mesenteric and crural conduit arteries is completely blocked by nifedipine, which seems to imply no role for Na/Ca exchange [150, 151]. However, since nifedipine can also block Na/Ca exchange [169] these results cannot discount a role for significant Na/Ca exchange in arteries contracted by digoxin.

Peripheral vascular resistance is determined by the tone of resistance arteries with diameters in the range of 4 to 400 μm [170] and for Blaustein's hypothesis to have credence, Na/Ca exchange must be present in vascular smooth muscle cells from these blood vessels. In the 1980s, Mulvany's group provided compelling evidence that neither reduction of the transplasmalemmal sodium gradient by Na/K-ATPase inhibition with ouabain nor incubation in low sodium solution (nor both together) raised the tone of resting isolated rat mesenteric resistance arteries [171]. The implication being that Na/Ca exchange is not an important transporter in small arteries [172]. We have shown, however, that incubation of human subcutaneous resistance arteries in either ouabain at a concentration of 10 nmol/liter and above or low sodium will cause concentration-dependent constriction which persists in the presence of diltiazem [91]. We have also found in these small human arteries that low extracellular sodium concentration will elevate free intracellular calcium concentration [173]. It may reasonably be argued that the resting resistance artery is a poor model of the situation *in vivo* when arteries do have some intrinsic tone, but, ouabain also potentiates the tone of submaximally precontracted human [91] and rat [153] resistance arteries. The precise mechanism for the increase in tone in the submaximally precontracted arteries may include a role for calcium influx through potential-dependent channels following

ouabain-induced depolarization but reverse mode Na/Ca exchange (that is, Na^+ out, Ca^{2+} in) cannot be discounted [163, 164].

The endothelial cell

The discovery of the endothelial cell layer as a source of potent vasoconstrictor and vasodilator substances has lead to its recognition as an important determinant of vasomotor tone. Endothelium-derived relaxing factor or nitric oxide (NO) is synthesized enzymatically from L-arginine by a calcium and NADPH dependent enzyme [174]. Using inactive analogues of L-arginine which block the synthesis of NO, it has been demonstrated that tonic release of NO from endothelial cells maintains the circulation of experimental animals [175] and the human forearm [176] in a state of active vasodilation. Ouabain inhibits endothelium-dependent relaxation in canine femoral arteries [177] and rat aorta [178], but has no effect on canine coronary [179, 180] or rabbit ear arteries [181]. In isolated human subcutaneous resistance arteries, we found that ouabain in concentrations of 100 pmol/liter and above caused concentration-dependent inhibition of endothelium-dependent relaxation [92], but interestingly had no effect on relaxation induced by sodium nitroprusside, a NO donor. Our results imply that sodium pump inhibition affects synthesis or release of NO. To our surprise, however, using forearm venous plethysmography, ouabain did not inhibit endothelium-dependent vasodilation induced by carbachol or bradykinin [182] though, as expected, endothelium-independent vasodilation by sodium nitroprusside was unaffected [147, 183]. The explanation for the discrepancy between our *in vitro* and *in vivo* findings remains uncertain but may either be due to a relative increase in the local concentration of the infused vasodilator resulting from ouabain-induced reduction in forearm blood flow or, alternatively, may reflect differences in Na/K-ATPase sensitivity to ouabain between the endothelial cells of skeletal muscle and subcutaneous vascular beds.

Endothelial cells also produce endothelin, a highly potent vasoconstricting peptide which has been implicated as a determinant of blood pressure [183], although the correlation may only reflect end organ damage. A physiological role for endothelin remains to be identified but incubation of cultured bovine pulmonary endothelial cells in a fraction of human urine with sodium pump inhibitory activity gave rise to a dose-dependent increase in endothelin secretion which was not seen with ouabain [184]. More recently, Badr's group [185] has demonstrated increased endothelin production and target cell response in tissue from Dahl-S rats exposed to ouabain as opposed to Dahl-R rats. Thus, once again endogenous sodium pump inhibitory activity has been implicated in the development of hypertension in Dahl-S rats [5]. It will be interesting to see whether a relationship between endogenous sodium pump inhibitory activity and endothelin levels can be established in EHT.

Effect of endogenous "ouabain" and "digoxin" on vascular contractility

Comprehensive investigation of the vasoreactivity of the two candidate Na/K-ATPase inhibitors isolated from human urine [50] and plasma [49] has not been carried out, largely due to the small quantities of active material available. Goto's urinary digoxin-like factor inhibits ^{86}Rb uptake increases calcium influx and decreases calcium efflux in cultured rat aortic smooth muscle cells [186]. However, experiments to ascertain the effects of their compound on vascular contractility were not conducted. In contrast, Hamlyn's "ouabain" in high concentration (170 nmol/liter) augments histamine-induced constriction of guinea pig aortic rings, but is without effect on resting tone, although experiments involving prolonged exposure were not carried out. In addition, "ouabain" causes concentration-dependent (at 85 and 170 nmol/liter) inotropy in repetitively stimulated atria [187]. Although prolonged digoxin administration in humans does not lead to a hypertensive response [152], prolonged ouabain ingestion by rats has recently been shown to increase blood pressure [90].

Two other lines of indirect evidence suggest a role for cardiac glycosides in the control of peripheral vascular resistance. A recent study has shown that the dilator response to intra-arterial potassium at low concentrations (leading to stimulation of the sodium pump) is blunted in saline-expanded subjects [188]. This might imply that salt-loading *per se*, perhaps through an endogenous sodium pump inhibitor, alters the regulation of vascular tone. This study is consistent with the conclusions made in some of the earliest studies in this field [189, 190] which showed a blunted dilator response to potassium in patients with essential hypertension. Although never proven to be due to a circulating sodium pump inhibitor, several early studies have shown that serum from hypertensive subjects has a constrictor effect on vascular smooth muscle when compared to that of normotensives [191–195].

Effect of progesterone derivatives and bufodienolides on vascularcontractility

Some derivatives of progesterone have been considered as likely candidates for the endogenous sodium pump inhibitor [95]. Although all inhibit isolated Na/K-ATPase, their functional activity in whole tissue depends on the configuration of the steroid nucleus [95]. Planar molecules demonstrate no inotropic or pressor activity [196, 197], whereas non-planar molecules (same conformation as cardiac glycosides) are inotropic in the isolated guinea pig heart [196], potentiate tone of submaximally precontracted human resistance arteries and inhibit endothelium-dependent relaxation [197]. To explain the dissociation between sodium pump inhibitory activity and lack of inotropy, LaBella et al have suggested that in addition to their ability to inhibit the sodium pump, the planar molecules can enter the cell and occupy an undefined receptor which prevents the usual intracellular accumulation of calcium which accompanies such inhibition [95].

The bufodienolides, which are potent inhibitors of the sodium pump, have been isolated from various animal [107, 108] and human tissues [109]. Bufalin, a commercially available bufodienolide, has been shown to block KCl-induced vasodilation, potentiate norepinephrine-induced vasoconstriction, increase blood pressure and cardiac contractility in the anesthetized dog [198] and rat [199]. Similarly, bufalin in concentrations of 1 nmol/liter and above potentiates the tone of submaximally precontracted isolated human resistance arteries and inhibits endothelium-dependent relaxation [197].

Conclusion

The evidence presented above demonstrates clearly that this subject is still very much alive. A change in direction towards the

identification of an inhibitor of active sodium transport and away from a natriuretic compound has focused the search. The main aim must be the identification of an endogenous ligand for the cardiac glycoside receptor on Na/K-ATPase and that this substance should inhibit active sodium transport. There is now wide appreciation of the problems involved in the assays of Na/K-ATPase activity. As a result, the emphasis has moved to complete purification and characterization of compounds which will inhibit Na/K-ATPase in several different assays. The most exciting development has been that of the positive identification of "ouabain" and the evidence that ouabain has a pressor action in human vascular tissue. Disappointingly, more than two years since the identification of "ouabain," there is still no evidence that it is elevated in EHT. However, the identification of a compound very similar to ouabain in the rat hypothalamus and the demonstration in one study that it is associated with hypertension, suggest that a family of ouabain-like cardiac glycosides which may be implicated in hypertension, exists in mammals. Despite the positive identification of a number of compounds, there is no room for complacency and the search for similar substances should be continued.

The possibility that the hypothalamus may be the source of a sodium pump inhibitor is of particular interest in the context of the increasing evidence that introduction of a Na/K-ATPase inhibitor into the hypothalamus may raise the systemic blood pressure by a central mechanism, although the effector pathway remains obscure. We suggest that this is likely to be one of the most rewarding areas of future research.

Now that there is little doubt that endogenous digoxin-like inhibitors of sodium transport exist, it is of prime importance that their physiological and pathophysiological roles are carefully elucidated. EHT is a disease affecting 10 to 15% of the world's population and the link between these substances, salt intake and vascular tone, must be pursued with increasing vigor.

ROBIN G. WOOLFSON, LUCILLA POSTON, AND HUGH E. DE WARDENER
London, England, United Kingdom

Reprint requests to Robin G. Woolfson, Department of Nephrology, Institute of Urology and Nephrology, University College London Medical School, 48 Riding House Street, London W1N 8AA, England, United Kingdom.

References

1. DE WARDENER HE, MILLS IH, CLAPHAM WF, HAYTER CJ: Studies on the efferent mechanism of the sodium diuresis which follows the administration of intravenous saline in the dog. *Clin Sci* 21:249–258, 1961
2. DE WARDENER HE, CLARKSON EM: Concept of natriuretic hormone. *Physiol Rev* 65:658–759, 1985
3. FLYNN TG, DE BOLD ML, DE BOLD AJ: The amino acid sequence of an atrial peptide with potent diuretic and natriuretic properties. *Biochem Biophys Res Commun* 117:859–865, 1983
4. SAGNELLA GA, NOLAN DA, SHORE AC, MACGREGOR GA: Effects of synthetic atrial natriuretic peptides on sodium-potassium transport in human erythrocytes. *Clin Sci* 69:223–226, 1985
5. DAHL LK, KNUDSEN KD, IWAI J: Humoral transmission of hypertension: Evidence from parabiosis. *Circ Res* 1(Suppl 24):21–33, 1969
6. HADDY FJ, OVERBECK HW: The role of humoral agents in volume expanded hypertension. *Life Sci* 19:935–948, 1976
7. BLAUSTEIN MP: Sodium ions, calcium ions, blood pressure regulation and hypertension: A reassessment and a hypothesis. *Am J Physiol* 232:C165–C173, 1977
8. DE WARDENER HE, MACGREGOR GA: Dahl's hypothesis that a saliuretic substance may be responsible for a sustained rise in arterial pressure—Its possible role in essential hypertension. *Kidney Int* 18:1–9, 1980
9. POSTON L, SEWELL RB, WILKINSON SP, RICHARDSON PJ, WILLIAMS R, CLARKSON EM, MACGREGOR GA, DE WARDENER HE: Evidence for a circulating sodium transport inhibitor in essential hypertension. *Br Med J* 282:847–849, 1981
10. MACGREGOR GA, FENTON S, ALAGHBAND-ZADEH J, MARKANDU N, ROULSTON JE, DE WARDENER HE: Evidence for a raised concentration of a circulating sodium transport inhibitor in essential hypertension. *Br Med J* 283:1355–1357, 1981
11. HAMLYN JM, RINGEL R, SCHAEFFER J, LEVINSON PD, HAMILTON BP, KOWARSKI AA, BLAUSTEIN MP: A circulating inhibitor of (Na^+ + K^+)ATPase associated with essential hypertension. *Nature* 300:650–652, 1982
12. MORETH K, KUSKE R, RENNER D, SCHONER W: Blood pressure in essential hypertension correlates with the concentration of a circulating inhibitor of the sodium pump. *Klin Wochenschr* 64:239–244, 1986
13. GRAY HH, HILTON PJ, RICHARDSON PJ: Effect of serum from patients with essential hypertension on sodium transport in normal leucocytes. *Clin Sci* 70:583–586, 1986
14. WECHTER WJ, BENSKAS EJ: Natriuretic hormones. *Prog Drug Res* 34:231–260, 1990
15. GOTO A, YAMADA K, YAMADA N, YOSHIOKA M, SUGIMOTO T: Physiology and pharmacology of endogenous digitalis-like factors. *Pharmacol Rev* 44:377–399, 1992
16. LICHTSTEIN D, SAMUELOV S, GATI I, WECHTER WJ: Digitalis-like compounds in animal tissues. *J Basic Clin Physiol Pharmacol* 3:269–292, 1992
17. LUFT FC, ARONOFF GR, FINEBERG NS, WEINBERGER MH: Effects of oral calcium, potassium, digoxin and nifedipine on natriuresis in normal humans. *Am J Hypertens* 2:14–19, 1991
18. HYMAN AL, JAQUES WE, HYMAN ES: Observations on the direct effect of digoxin on renal excretion of sodium and water. *Am Heart J* 52:592–608, 1956
19. CLARKSON EM, DE WARDENER HE: Observations on a low molecular weight natriuretic and Na-K-ATPase inhibiting material in urine. *Clin Exp Hyper Theor Pract* A7(5&6):673–683, 1985
20. BENAKSAS EJ, MURRAY ED, RODGERS CL, PHAM T, BIGORNIA AE, DE WIND SA, GIEBEL R, BRUBACHER ES, WECHTER WJ: Endogenous natriuretic factors 1: Sodium pump inhibition does not correlate with natriuretic or pressor activities from uremic urine. *Life Sci* 52:1045–1054, 1993
21. VASDEV S, JOHNSON E, LONGERICH L, PRABHAKARAN VM, GAULT MH: Plasma endogenous digitalis-like factors in healthy individuals and in dialysis dependent and kidney transplant patients. *Clin Nephrol* 27:169–174, 1987
22. LONGERICH L, JOHNSON R, BRENT D, GAULT MH: Plasma conjugated androgens in a dialysis dependent male as immunoreactive digitalis-like factors. *Clin Nephrol* 34:72–78, 1990
23. SOHN H-J, STOKES GS, JOHNSTON H: An Na, K ATPase inhibitor from ultrafiltrate obtained by hemodialysis of patients with uremia. *J Lab Clin Med* 120:264–271, 1992
24. WENZEL M, HEIDRICH E, KIRCH U, MORETH-WOLFRAT K, WIZEMANN V, SCHONER W: Increased concentrations of a circulating sodium pump inhibitor in essential hypertension and uraemia and its partial purification from hemofiltrate. *Physiol Bohemoslov* 39:79–85, 1990
25. GOTTLEIB SS, ROGOWSKI AC, WEINBERG M, KRICHTEN CM, HAMILTON BP, HAMLYN JM: Elevated concentrations of endogenous ouabain in patients with congestive heart failure. *Circulation* 86:420–425, 1992
26. BAGROV AY, FEDOROVA OV, MASLOVA MN, ROUKOYATKINA NI, UKHANOVA MV, ZHABKO EP: Endogenous plasma Na,K-ATPase inhibitory activity and digoxin like immunoreactivity after acute myocardial infarction. *Cardiovasc Res* 25:371–377, 1991
27. SEWELL RB, HUGHES RD, POSTON L, WILLIAMS R: Effects of serum from patients with fulminant hepatic failure on leucocyte sodium transport. *Clin Sci* 63:237–242, 1982
28. LINGREL JB: Na,K-ATPase: Isoform structure, function, and expression. *J Bioenergetics Biomembr* 24:263–270, 1992

29. MAIXENT JM, FENARD S, KAWAMOTO RM: Tissue localization of active Na,K ATPase isoenzymes by determination of their profile inhibition with ouabain, digoxin, digitoxigenin and LND 796, a new aminosteroid cardiotonic. *J Recept Res* 11:687–698, 1991
30. BALZAN S, MONTALI U, GENOVESI-EBERT A, BIVER P, FANTONI M, GHIONE S: Comparison between endogenous digoxin-like immunoreactivity and ^{86}Rb uptake by erythrocytes in extracts of human plasma. *Clin Sci* 77:375–381, 1989
31. DEVYNCK MA, PERNOLLET MG, ROSENFELD JB, MEYER P: Measurement of digitalis-like compound in plasma: Application in studies of essential hypertension. *Br Med J* 287:631–634, 1983
32. BOURGOIGNIE JJ, ESPINEL C, KLAHR S, BRICKER NS: A natriuretic factor in the serum of patients with chronic uremia. *J Clin Invest* 51:1514–1527, 1972
33. GOTO A, YAMADA K, ISHII M, SUGIMOTO T: How best to detect endogenous digitalis-like factor? *Clin Chem* 34:2392–2393, 1988
34. GRAVES SW, WILLIAMS GH: Endogenous digitalis-like natriuretic factors. *Annu Rev Med* 38:433–444, 1987
35. TAL DM, KATCHALSKY S, LICHSTEIN D, KARLISH SJD: Endogenous "ouabainlike" activity in bovine plasma. *Biochem Biophys Res Commun* 135:1–8, 1986
36. HAMLYN JM, HARRIS DW, LUDENS JH: Digitalis-like activity in human plasma. *J Biol Chem* 264:7395–7404, 1989
37. APELL H-J, MARCUS MM: $(Na^+ + K^+)$-ATPase in artificial lipid vesicles: influence of the concentration of mono- and divalent cations on the pumping rate. *Biochem Biophys Acta* 862:254–264, 1986
38. CANTLEY L, JOSEPHSON CL, WARNER M, YANAGISAWA M, LECHENE C, GUIDOTTI G: Vanadate is a potent (Na,K)-ATPase inhibitor found in ATP derived from muscle. *J Biol Chem* 252:7421–7423, 1977
39. PATRICK J, MICHAEL J, GOLDEN MN, GOLDEN BE, HILTON PJ: Effect of zinc on leucocyte sodium transport *in vitro*. *Clin Sci Mol Med* 54:585–587, 1978
40. NG Y-C, AKERA T, HAN C-S, BRASELTON WE, KENNEDY RH, TEMMA K, BRODY TM, SATO PH: Ascorbic acid: An endogenous inhibitor of isolated Na^+,K^+ ATPase. *Biochem Pharmacol* 34:2525–2530, 1985
41. KARIYA K, SANO H, YAMANSISHI J, SAITO K, FURUTA Y, FUKUZAKI H: A circulating Na^+-K^+ ATPase inhibitor, erythrocyte sodium transport and hypertension in patients with chronic renal failure. *Clin Exp Theor Pract* A8:167–183, 1986
42. HAMLYN JM, ASHEN MD, FORREST B, ROGOWSKI AC, WHITE RJ: Species sensitivity of the sodium pump to a circulating ouabain-like inhibitor in acute hypervolemia and DOCA hypertension: Comparison with ouabain. *Prog Biochem Pharmacol* 23:22–34, 1988
43. CLARKSON EM, KOUTSAIMINIS KG, DAVIDMAN M, DUBOIS M, PENN WP, DE WARDENER HE: The effect of brain extracts on urinary sodium excretion of the rat and intracellular sodium concentration of renal tubule fragments. *Clin Sci Mol Med* 47:210–213, 1974
44. ROELOFSEN B: The (non) specificity in the lipid-requirement of calcium- and (sodium plus potassium)-transporting adenosine triphosphatase. *Life Sci* 29:2235–2247, 1981
45. GIRAUD F, CLARET M, BRUCKDORFER KR, CHAILLEY B: The effects of membrane lipid order and cholesterol on the internal and external cationic sites of the Na-K pump in erythrocytes. *Biochim Biophys Acta* 647:249–258, 1981
46. MATSUDA T, IWATA H: Difference in phospholipid dependence between two isoenzymes of brain $(Na^+ + K^+)$-ATPase. *Biochim Biophys Acta* 860:620–625, 1986
47. NG LL, HOCKADAY TDR: Non-esterified fatty acids may regulate human leucocyte sodium pump activity. *Clin Sci* 71:737–742, 1986
48. SCHMALZING G, KUTSCHERA P: Modulation of ATPase activities of human erythrocyte membranes by free fatty acids or phosholipase A_2. *J Membr Biol* 69:6576, 1982
49. HAMLYN JM, BLAUSTEIN MP, BOVA S, DU CHARME DW, HARRIS DW, MANDEL F, MATHEWS WR, LUDENS JH: Indentification and characterization of a ouabain-like compound from human plasma. *Proc Natl Acad Sci USA* 88:6259–6263, 1991
50. GOTO A, ISHIGURO T, YAMADA K, ISHII M, YOSHIOKA M, EGUCHI C, SHIMORA M, SUGIMOTO T: Isolation of urinary digitalis-like factor indistinguishable from digoxin. *Biochem Biophys Res Commun* 173: 1093–1101, 1990
51. CLOIX J-F, CRABOS M, GRICHOIS M-L, GUICHENEY P, MEYER P: Endogenous Na^+-pump inhibitor extracted from human urine: Further biochemical studies. *J Hypertens* 4:S353–S354, 1986
52. LICHSTEIN D, SAMUELOV S: Endogenous "ouabain-like" activity in rat brain. *Biochem Biophys Res Commun* 96:1518–1523, 1980
53. INAGAMI T, TAMURA M: Purification and characterization of specific endogenous ouabainlike substance from bovine adrenal. *Am J Med Sci* 295:400–405, 1988
54. MACGREGOR GA, FENTON S, ALAGHBAND-ZADEH J, MARKANDU ND, ROULSTON JE, DE WARDENER HE: An increase in a circulating inhibitor of Na-K-dependent ATPase: A possible link between salt intake and the development of essential hypertension. *Clin Sci* 61:17S–20S, 1981
55. SOLDIN SJ: Digoxin-issues and controversies. *Clin Chem* 32:5–12, 1986
56. BESCH HR, HUFFERD S, LAKE M, HURWITZ R, WATANABE AM: False elevation of apparent digoxin levels in plasma of premature infants. *Clin Chem* 22:1168, 1976
57. VALDES R, GRAVES SW, BROWN BA, LANDT M: Endogenous substance in newborn infants causing false positive digoxin measurements. *J Pediatr* 102:947–950, 1983
58. DIAMANDIS EP, PAPANASTASIOU-DIAMANDI A, SOLDIN SJ: Digoxin immunoreactivity in cord and maternal serum and placental extracts. Partial characterization of immunoreactive substances by high-performance liquid chromatography and inhibition of Na^+,K^+-ATPase. *Clin Biochem* 18:49–55, 1985
59. MORRIS JF, MCEACHERN MD, POSTON L, SMITH SE, MULVANY MJ, HILTON PJ: Evidence for an inhibitor of leucocyte sodium transport in the serum of neonates. *Clin Sci* 73:291–297, 1987
60. SECCOMBE DW, PUDEK MR, HUMPHRIES KH, MATTHEWSON B, TAYLOR GP, JACOBSON BE, WHITFIELD MP: A study into the nature and organ source of digoxin-like immunoreactive substance in the perinatal period. *Biol Neonate* 56:136–146, 1989
61. BALZAN S, GHIONE S, BIVER P, CLERICO A, MONTALI U: Correlation between endogenous digoxin-like immunoreactivity and ^3H-ouabain displacement on erythrocyte membranes in extracts of human plasma. *Clin Biochem* 19:311–314, 1986
62. SHUN YUN W, HO CS, PANESAR NS, SWAMINATHAN RS: The contribution of steroids to digoxin-like immunoreactivity in cord blood. *Ann Clin Biochem* 29:337–342, 1992
63. GRAVES SW, VALDES R, BROWN BA, KNIGHT AB, CRAIG HR: Endogenous digoxin-immunoreactive substance in human pregnancies. *J Clin Endocrinol Metabol* 58:748–751, 1984
64. VALDES R, GRAVES SW, KNIGHT AB, CRAIG HR: Endogenous digoxin immunoactivity is elevated in hypertensive pregnancy. *Prog Clin Biol Res* 192:229–232, 1985
65. POSTON L, MORRIS JF, WOLFE CD, HILTON PJ: Serum digoxin-like substances in pregnancy-induced hypertension. *Clin Sci* 77:189–194, 1989
66. HICKS JM, BRETT EM: Falsely increased digoxin concentrations in samples from neonates and infants. *Ther Drug Monitor* 6:461–464, 1984
67. CHESALOW FI, BRETHEN SL: Characterization of digoxin-like materials in human cord serum. *Ann NY Acad Sci* 595:212–221, 1990
68. KELLY RA, O'HARA DS, CANESSA ML, MITCH WE, SMITH TW: Characterization of digitalis-like factors in human plasma: Interaction with Na/K ATPase and cross reactivity with cardiac glycoside-specific antibodies. *J Biol Chem* 260:11396–11405, 1985
69. YAMADA K, GOTO A, ISHII M, YOSHIOKA M, SUGIMOTO T: Dissociation of digoxin-like immunoreactivity and Na^+,K^+-ATPase inhibitory activity in rat plasma. *Experientia* 44:992–993, 1988
70. HNATOWICH M, LABELLA F: Endogenous digitalis-like factors: *in vitro* comparison of biological and immunological activities of peptides and steroid candidates. *Eur J Pharmacol* 106:567–575, 1985
71. MONTALI U, BALZAN S, GHIONE S: Purification of endogenous digitalis-like factor(s) from cord blood of neonate by immunoaffinity chromatography. *Biochem Intern* 25:853–859, 1991
72. BALZAN S, MONTALI U, BIVER P, GHIONE S: Digoxin-binding antibodies reverse the effect of endogenous digitalis-like compounds on Na,K-ATPase in erythrocytes. *J Hypertens* 9:S304–S305, 1991
73. HUANG BS, HARMSEN E, YU H, LEENEN FHH: Brain ouabain-like activity and the sympathoexcitatory and pressor effects of central sodium in rats. *Circ Res* 71:1059–1066, 1992

74. GOODLIN RC: Antidigoxin antibodies in eclampsia. *N Engl J Med* 318:518–519, 1988
75. TAMURA M, KUWANO H, KINOSHITA T, INAGAMI T: Identification of linoleic and oleic acids as endogenous Na^+,K^+-ATPase inhibitors from acute volume-expanded hog plasma. *J Biol Chem* 260:9672–9677, 1989
76. KELLY RA, O'HARA DS, MITCH WE, SMITH TW: Identification of Na,K-ATPase inhibitors in human plasma as non-esterified fatty acids and lysophospholipids. *J Biol Chem* 261:11704–11711, 1986
77. TAL DM, YANUCK MD, HALL GV, KARLISH SJD: Identification of Na^+/K^+-ATPase inhibitors in bovine plasma as fatty acids and hydrocarbons. *Biochim Biophys Acta* 985:55–59, 1989
78. GODFRIEND TL, BALL DB, WEINBERGER MH, MOORE TJ, WEDER AB, EGAN BE: Salt loads raise plasma fatty acids and lower insulin. *Hypertension* 17:958–964, 1991
79. LICHSTEIN D, SAMUELOV S, GATI I, FELIX AM, GABRIEL TF, DEUTSCH J: Identification of 11,13-dihydroxy-14-octadecaenoic acid as a circulating Na^+/K^+ ATPase inhibitor. *J Endocrinol* 128:71–78, 1991
80. OISHI K, ZHENG B, KUO JF: Inhibition of Na,K ATPase and sodium pump by protein kinase C regulators sphingosine, lysophosphatidylcholine, and oleic acid. *J Biol Chem* 265:70–75, 1990
81. HANSEN O: Characterization of fatty acid interaction with ouabain and vanadate binding to (Na^+ + K^+)-activated ATPase. *Biochim Biophys Acta* 986:130–134, 1989
82. FREDRICKSON DS, GORDON RS: Transport of fatty acids. *Physiol Rev* 38:585–630, 1958
83. LAU BWC, VALDES R: Criteria for identifying endogenous compounds as digoxin-like immunoreactive factors in humans. *Clin Chim Acta* 175:67–78, 1988
84. HAMLYN JM, SCHENDEN JA, ZYREN J, BACZYNSKJ L: Purification and characterization of digitalis-like factors from human plasma. *Hypertension* 10(Suppl 1):71–77, 1987
85. TAMURA M, HARRIS TM, HIGASHIMORI K, SWEETMAN BJ, BLAIR IA, INAGAMI T: Lysophosphatidylcholines containing polyunsaturated fatty acids were found as Na^+,K^+-ATPase inhibitors in acutely volume expanded hog. *Biochemistry* 26:2797–2806, 1987
86. DASGUPTA A, MALIK S, AHMAD S, KENNY M: Mass spectrometry studies of a novel digoxin-like substance (DLIS-2) isolated from human plasma ultrafiltrate. *Biochem Biophys Res Commun* 152:1435–1440, 1988
87. LUDENS JH, CLARK MA, DUCHARME DW, HARRIS DW, LUTZKE BS, MANDEL F, MATHEWS WR, SUTTER DM, HAMLYN JM: Purification of an endogenous digitalislike factor from human plasma for structural analysis. *Hypertension* 17:923–929, 1991
88. MATHEWS WR, DUCHARME DW, HAMLYN JM, HARRIS DW, MANDEL F, CLARK MA, LUDENS JH: Mass spectral characterisation of an endogenous digitalislike factor from human plasma. *Hypertension* 17:930–935, 1991
89. HARRIS DW, CLARK MA, FISHER JF, HAMLYN JM, KOLBASA KP, LUDENS JH, DUCHARME DW: Development of an immunoassay for endogenous digitalislike factor. *Hypertension* 17:936–943, 1991
90. HAMLYN JM, MANUNTA P: Ouabain, digitalis-like factors and hypertension. *J Hypertens* 10(Suppl 7):S99–S111, 1992
91. WOOLFSON RG, HILTON PJ, POSTON L: Effects of ouabain and low sodium on contractility of human resistance arteries. *Hypertension* 15:583–590, 1990
92. WOOLFSON RG, POSTON L: Effect of ouabain on endothelium-dependent relaxation of human resistance arteries. *Hypertension* 17:619–625, 1991
93. MALAWISTA I, DAVIDSON EA: Isolation and identification of rhamnose from rabbit skin. *Nature* 192:871–872, 1961
94. ROSENFELD EL, WIEDERSCHEIN GY: The metabolism of L-rhamnose in animal tissues. *Bull Soc Chim Biol* 47:1433–1440, 1965
95. LABELLA FS, TEMPLETON JF, SASHI KUMAR VP, BOSE D: Progesterone derivatives that bind to the digitalis receptor. *TIPS* 10:11–14, 1989
96. WEILAND J, SCHOENFELD W, MENKE K-H, REPKE KRH: Interaction of progesterone derivatives with the digitalis target enzyme: Impact of glycosidation on inhibitory action. *Pharmacol Res* 23:27–32, 1991
97. KELLY RA, SMITH TW: Is ouabain the endogenous digitalis? *Circulation* 86:694–696, 1992
98. SAGNELLA GA, MACGREGOR GA: Characteristics of Na/K ATPase inhibitors in extracts of tea. *Am J Clin Nutr* 40:46–49, 1984
99. KELLY RA: Excretion of endogenous digitalis-like factors. *Am J Physiol* 251:H205–H209, 1986
100. LUDENS JH, CLARK MA, ROBINSON FG, DUCHARME DW: Rat adrenal cortex is a source of a circulating ouabainlike compound. *Hypertension* 19:721–724, 1992
101. GOTO A, YAMADA K, ISHII M, YOSHIOKA M, ISHIGURO T, EGUCHI C, SUGIMOTO T: Purification and characterization of human urine derived digitalislike factor. *Biochem Biophys Res Commun* 154:847–853, 1988
102. GOTO A, YAMADA K, ISHII M, YOSHIOKA M, ISHIGURO T, EGUCHI C, SUGIMOTO T: Presence of digitalis-like factor in mammalian plasma. *Biochem Biophys Res Commun* 152:322–327, 1988
103. GOTO A, YAMADA K, ISHII M, SUGIMOTO T: Release of endogenous digitalis-like factor with sodium loading. *N Eng J Med* 320:124–125, 1989
104. MASUGI F, OGIHARA T, HASEGAWA T, KUMAHARA Y: Ouabain and non-ouabain like factors in plasma of patients with essential hypertension. *Clin Exp Hypertens* A9:1233–1242, 1987
105. MASUGI F, OGIHARA T, SAKAGUCHI K, KUMAHARA Y: Partial purification and properties of a plasma ouabain-like inhibitor of Na^+,K^+-ATPase in patients with essential hypertension. *J Hypertens* 6:S351–S353, 1988
106. GOTO A, ISHIGURO T, YAMADA K, ISHII M, YOSHIOKA M, EGUCHI C, SHIMORA M, SUGIMOTO T: Isolation of a urinary digitalislike factor indistinguishable from digoxin. *Biochem Biophys Res Commun* 173: 1093–1101, 1990
107. FLIER JS, MARATOS-FLIER E, PALLOTA JA, MCISAAC D: Endogenous digitalis-like activity in the plasma of the toad *Bufo marinus*. *Nature* 279:341–343, 1979
108. AKIZAWA T, YASUHARA T, KANO R, NAKAJIMA T: Novel polyhydroxylated cardiac steroids in the nuchal glands of the snake, *Rhabdophis tigrinus*. *Biomed Res* 6:437–441, 1985
109. LICHTSTEIN D, GATI I, SAMUELOV S, BERSON D, ROZENMAN Y, LANDAU L, DEUTSCH J: Identification of digitalis-like compounds in human cataractous lenses. *Eur J Biochem* 216:261–268, 1993
110. SEN A, JAGGI FJ, TSAI TYR, WEISNER K: A simple and efficient synthesis of bufalin. *J Chem Soc Chem Commun* 21:1213–1214, 1982
111. PAMNANI M, HUOT S, BUGGY J, CLOUGH D, HADDY F: Demonstration of a humoral inhibitor of the Na-K pump in some models of experimental hypertension. *Hypertension* 3(Suppl II):96–101, 1981
112. BUGGY J, HUOT S, PAMNANI M, HADDY F: Periventricular forebrain mechanisms for blood pressure regulation. *Fed Proc* 43:25–31, 1984
113. HUOT SJ, PAMNANI MB, CLOUGH DL, BUGGY J, BRYANT HJ, HARDER DR, HADDY FJ: Sodium-potassium pump activity in reduced renal mass hypertension. *Hypertension* 5(Suppl 1):94–100, 1983
114. SONGU-MIZE E, BEALER SL, CALDWELL RW: Effect of DOCA-salt treatment duration and anteroventral third ventricle lesions in plasma-borne sodium pump inhibitor in rats. *J Hypertens* 5:461–467, 1987
115. JANDHYALA BS, ANSARI AF: Elevation of sodium levels in the cerebral ventricle of anaesthetized dogs triggers the release of an inhibitor of ouabain-sensitive sodium, potassium-ATPase into the circulation. *Clin Sci* 70:103–110, 1986
116. KATAFUCHI T, OOMURA Y, MARUYAMA T, AKAIKE N: Hypothalamus and sodium-potassium pump activity in skeletal muscles of DOCA-hypertensive rats. *Am J Physiol* 253:R396–R401, 1987
117. JANDHYALA BS, LOKHANDWALA MF, KIVLIGHIN SD, ANSARI AF, DE FEO ML: Intracisternal administration of pergolyde, a dopamine receptor agonist, triggers the release of an inhibitor of ouabain-sensitive sodium, potassium-dependent adenosine triphosphatase and enhances vascular reactivity. *Clin Sci* 73:183–188, 1987
118. SONGU-MIZE E, BEALER SL, HASSID AI: Centrally administered ANF promotes appearance of a circulating sodium pump inhibitor. *Am J Physiol* 258:H1655–H1659, 1990
119. YAMADA K, GOTO A, HUI C, YAGI N, SUGIMOTO T: Effects of Fab fragments of digoxin antibody on the natriuresis and increase in blood pressure induced by intracerebroventricular infusion of hypertonic saline solution in rats. *Clin Sci* 82:625–630, 1992
120. HUANG BS, LEENEN FHH: Brain ouabain and central effects of

dietary sodium in spontaneously hypertensive rats. *Circ Res* 70:430–437, 1992

121. Iyoda I, Takahashi H, Lee LC, Okajima H, Inoue A, Sasaki S, Takeda K, Yoshimura M, Iiichi H: Cardiovascular and sympathetic response to ouabain injected into the hypothalamus of rats. *Cardiovasc Res* 20:294–298, 1986
122. Yamada H, Ihara N, Sano Y: Morphological evidence of endogenous digitalis-like substance (EDLS) in the rat and macaque hypothalamus using digoxin-immunochemistry. *Endocrinol Jpn* 34:319–323, 1987
123. Yamada H, Ihara N, Takahashi H, Yoshimura M, Sano Y: Distribution of the endogenous digitalis-like substance (EDLS) containing neurons labelled by digoxin antibody in hypothalamus and three circumventricular organs of dog and macaque. *Brain Res* 584:237–243, 1992
124. Yamada H, Naruse M, Naruse K, Demura H, Takahashi H, Yoshimura M, Ochi J: Histological study on ouabain immunoreactivity in the mammalian hypothalamus. *Neurosci Lett* 141:143–146, 1992
125. Ihara N, Yuri K, Yamada H, Sano Y: Immunohistochemical studies on the distribution of endogenous digitalis-like substance (EDLS)-containing neurons in the rat hypothalamus, with special consideration on the possibility of their coexistence with posterior lobe hormones. *Arch Histol Cytol* 51:35–42, 1988
126. Mir MA, Chappell SP, Morgan K, Lewis MD, Scanlon MF, Lewis MD, Scanlon MF, Lewis MJ: Hypothalamic sodium transport inhibitor and vascular reactivity. *J Hypertens* 4:S233–S235, 1986
127. Cloix J-F, Crabos M, Wainer IW, Ruegg U, Seiler M, Meyer P: High yield purification of a urinary Na pump inhibitor. *Biochem Biophys Res Commun* 131:1234–1240, 1985
128. Goto A, Yamada K, Ishii M, Sugimoto T: Digitalis-like activity in human plasma: Relation to blood pressure and sodium balances. *Am J Med* 89:420–426, 1990
129. Haber E, Haupert GT: The search for a hypothalamic Na, K, ATPase inhibitor. *Hypertension* 9:315–324, 1987
130. Haupert GT, Carilli CT, Cantley LC: Hypothalamic sodium-transport inhibitor is a high affinity reversible inhibitor of Na-K-ATPase. *Am J Physiol* 247:F919–F924, 1984
131. Tymiak AA, Norman JA, Bolgar M, Didonato GC, Lee H, Parker WL, Lo L-C, Berova N, Nakanishi K, Haber E, Haupert GT: Physicochemical characterization of an ouabain isomer isolated from bovine hypothalamus. *Proc Natl Acad Sci USA* 90:8189–8193, 1993
132. Ferrandi M, Minotti E, Salardi S, Florio M, Bianchi G, Ferrari P: Ouabain-like factor in Milan hypertensive rats. *Am J Physiol* 263:F739–F748, 1992
133. de Wardener HE, MacGregor GA, Clarkson EM, Alaghband-Zadeh J, Bitensky L, Chayen J: Effect of sodium intake on ability of human plasma to inhibit renal Na,K adenosine triphosphatase *in vitro*. *Lancet* i:411–412, 1981
134. Fenton S, Clarkson E, MacGregor GA, Alaghband-Zadeh J, de Wardener HE: An assay of the capacity of biological fluids to stimulate renal glucose-6-phosphate dehydrogenase activity *in vitro* as a marker of their ability to inhibit sodium potassium dependent adenosine triphosphatase activity. *J Endocrinol* 94:99–110, 1982
135. Millett JA, Holland SM, Alaghband-Zadeh J, de Wardener HE: Na-K-ATPase-inhibiting and glucose-6-phosphate dehydrogenase-stimulating activity of plasma and hypothalamus of the Okamoto spontaneously hypertensive rat. *J Endocr* 108:69–73, 1986
136. Holland S, Millett J, Alaghband-Zadeh J, de Wardener HE, Ferrari P, Bianchi G: Cytochemically assayable Na,K-ATPase inhibition by Milan hypertensive rat plasma. *Hypertension* 9:498–503, 1987
137. Holland S, Millett J, Alaghband-Zadeh J, de Wardener HE, Pamnani M, Haddy F: Cytochemically detectable glucose-6-phosphate dehydrogenase-stimulating/Na-K-ATPase inhibiting activity of plasma and hypothalamus in reduced renal mass hypertension. *Am J Hypertens* 4:315–320, 1991
138. Alaghband-Zadeh J, Fenton S, Hancock K, Millett J, di Wardener HE: Evidence that the hypothalamus may be a source of a circulating Na/K-ATPase inhibitor. *J Endocrinol* 98:221–226, 1983
139. Wardener de HE, Holland S, Alaghband-Zadeh J, Morris HR, Panico M, Etienne A: A possible connection between an increased concentration of a cytochemically detectable substance in the hypothalamus of the spontaneously hypertensive rat and certain cerebral cholinergic disturbances. *J Cardiovasc Pharmacol* 22(Suppl 2):S109–S111, 1993
140. Gillis RA, Quest JA: The role of the nervous system in the cardiovascular effects of digitalis. *Pharmacol Rev* 31:19–97, 1979
141. Powis DA: Cardiac glycosides and autonomic neurotransmission. *J Auton Pharmacol* 3:127–154, 1983
142. Lorenz RR, Powis DA, Vanhoutte PM, Shepherd JT: The effects of acetylstrophanthidin and ouabain on the sympathetic adrenergic neuroeffector junction in canine vascular smooth muscle. *Circ Res* 47:845–854, 1980
143. Kranzhofer R, Haass M, Kurz T, Richardt G, Schomig A: Effect of digitalis glycosides on norepinephrine release in the heart. *Circ Res* 68:1628–1637, 1991
144. Bogdanski DF, Brodie BB: The effects of inorganic ions on the storage and uptake of ^{3}H-norepinephrine by rat heart slices. *J Pharamacol Exp Ther* 165:181–189, 1969
145. Iversen LL: Role of transmitter uptake mechanisms in synaptic neurotransmission. *Br J Pharmacol* 41:571–591, 1971
146. Pedrinelli R, Taddei S, Salvetti A: Sympathetic vasoconstriction as a mechanism of action of ouabain in forearm arterioles of hypertensive patients. *Clin Sci* 77:541–545, 1989
147. Mason DT, Braunwald E: Studies on digitalis. X. Effects of ouabain on forearm vascular resistance and venous tone in normal subjects and in patients with heart failure. *J Clin Invest* 43:532–543, 1964
148. Robinson BF, Phillips RJW, Wilson PN, Chiodoni PL: Effect of local infusion of ouabain on human forearm vascular resistance and on response to potassium and sodium nitroprusside. *J Hypertens* 1:165–169, 1983
149. Hulthen UL, Bolli P, Kiowski W, Buhler FR: Forearm vasoconstrictor response to ouabain: Studies in patients with mild and moderate essential hypertension. *J Cardiovasc Pharmacol* 6:S75–S81, 1984
150. Mikkelsen E, Andersson K-E, Pedersen OF: Effects of digoxin on isolated human peripheral arteries and veins. *Acta Pharmacol Toxicol* 45:249–256, 1979
151. Mikkelsen E, Andersson K-E, Pedersen OF: Effects of digoxin on isolated human mesenteric vessels. *Acta Pharmacol Toxicol* 45:25–31, 1979
152. Guthrie GP: Effect of digoxin on responsiveness to the pressor actions of angiotensin and norepinephrine in man. *J Clin Endocrinol Metab* 53:76–80, 1984
153. Aalkjaer C, Mulvany MJ: Effect of ouabain on tone, membrane potential and sodium efflux compared with [^{3}H]ouabain binding in rat resistace vessels. *J Physiol* 362:215–231, 1985
154. Mulvany MJ: Changes in sodium pump activity and vascular contraction. *J Hypertens* 3:429–436, 1985
155. Poston L: Endogenous sodium pump inhibitors: A role in essential hypertension? *Clin Sci* 72:647–655, 1987
156. Forrester TE, Alleyne GAO: Leucocyte electrolytes and sodium efflux rate constants in the hypertension of pre-eclampsia. *Clin Sci* 59:199s–201s, 1980
157. McMurray JA, Morgan DB: The sodium content of lymphocytes and mixed leucocytes in pregnancy and pregnancy induced hypertension. *Clin Exp Hypertens* B3:23–30, 1984
158. Reuter H, Seitz N: The dependence of calcium efflux from cardiac muscle on temperature and external ion composition. *J Physiol* 195:451–470, 1968
159. Baker PF, Blaustein MP, Hodgkin AL, Steinhardt RA: The influence of calcium on sodium efflux in squid axons. *J Physiol* 200:431–458, 1969
160. Reuter H, Blaustein MP, Haeusler G: Na-Ca exchange and tension development in arterial smooth muscle. *Phil Trans R Soc Lond* 265:87–94, 1973
161. Nicoll DN, Longoni S, Philipson KD: Molecular cloning and functional expression of the cardiac sarcolemmal Na^+-Ca^{2+} exchanger. *Science* 250:562–565, 1990
162. Blaustein MP: Sodium/calcium exchange and the control of contractility in cardiac muscle and vascular smooth muscle. *J Cardiovasc Pharmacol* 12(Suppl 5):S56–S68, 1988

163. ASHIDA T, BLAUSTEIN MP: Regulation of cell calcium and contractility in mammalian arterial smooth muscle: the role of sodium-calcium exchange. *Am J Physiol* 392:617–635, 1987
164. AARONSON PI, BENHAM CD: Alterations in $[Ca]_i$ mediated by sodium-calcium exchange in smooth muscle isolated from the guinea-pig ureter. *J Physiol (Lond)* 416:1–18, 1989
165. SATO K, AOKI K: Early and late contraction induced by ouabain in human umbilical arteries. *Br J Pharmacol* 103:1525–1529, 1991
166. KLEYMAN TR, CRAGOE EJ JR: Amiloride and its analogs as tools in the study of ion transport. *J Membr Biol* 105:1–21, 1988
167. FERNANDEZ-ALFONSO MS, SANCHEZ-FERRER CF, HERNANDEZ MC, MARIN JC: Na^+/Ca^{2+} exchange mediation in the ouabain-induced contraction in human placental vessels. *Gen Pharmacol* 23:439–444, 1992
168. MARIN JC, FERNANDEZ-ALFONSO MS, SANCHEZ-FERRER CF: Sodium pump activity and contractile effect of ouabain in human placental veins. *Eur J Pharmacol* 201:75–82, 1991
169. KACKZOROWSKI GJ, SLAUGHTER RS, KING VF, GARCIA ML: Inhibitors of sodium-calcium exchange: Identification and development of probes of transport activity. *Biochim Biophys Acta* 988:287–302, 1989
170. MULVANY MJ, AALKJAER C: Structure and function of small arteries. *Physiol Rev* 70:921–961, 1990
171. MULVANY MJ, AALKJAER C, PETERSEN TT: Intracellular sodium, membrane potential, and contractility of rat mesenteric small arteries. *Circ Res* 54:740–749, 1984
172. MULVANY MJ: Changes in sodium pump activity and vascular contraction. *J Hypertens* 3:429–436, 1985
173. AARONSON PI, POSTON L, WOOLFSON RG, SMIRNOV SV: Evidence for Na/Ca exchange in human resistance arteries. *Ann NY Acad Sci* 639:521–530, 1992
174. MONCADA S, PALMER RMJ, HIGGS EA: Nitric oxide: Physiology, pathophysiology and pharmacology. *Pharmacol Rev* 43:109–142, 1991
175. REES DD, PALMER RMJ, MONCADA S: Role of endothelium-derived nitric oxide in the regulation of blood pressure. *Proc Natl Acad Sci USA* 86:3375–3378, 1989
176. VALLANCE P, COLLIER JGT, MONCADA S: Effects of endothelium-derived nitric oxide on peripheral arteriolar tone in man. *Lancet* ii:997–1004, 1989
177. DE MEY JG, VANHOUTTE PM: Interaction between Na,K exchanges and the direct inhibitory action of acetylcholine on canine femoral arteries. *Circ Res* 48:826–835, 1980
178. RAPOPORT RM, SCHWARTZ K, MURAD F: Effects of Na,K-pump inhibitors and membrane depolarizing agents on acetylcholine-induced endothelium-dependent relaxation and cyclic GMP accumulation in rat aorta. *Eur J Pharmacol* 110:203–209, 1985
179. CHEN G, HASHITANI H, SUZUKI H: Endothelium-dependent relaxation and hyperpolarization of canine coronary artery smooth muscles in relation to the electrogenic Na-K pump. *Br J Pharmacol* 98:950–956, 1989
180. FELETOU M, VANHOUTTE PM: Endothelium-dependent hyperpolarization of canine coronary smooth muscle. *Br J Pharmacol* 93:515–524, 1988
181. SUZUKI H: The electrogenic Na-K pump does not contribute to endothelium-dependent hyperpolarization in the rabbit ear. *Eur J Pharmacol* 156:292–297, 1988
182. WOOLFSON RG, BENJAMIN N, TODD SD, POSTON L, RITTER JM: Ouabain and responses to endothelium-dependent vasodilators in the human forearm. *Br J Clin Pharmacol* 32:758–760, 1991
183. DAVENPORT AP, ASHBY MJ, EASTON P, ELLA S, BEDFORD J, DICKERSON C, NUNEZ DJ, CAPPER SJ, BROWN MJ: A sensitive radioimmunoassay measuring endothelin-like immunoreactivity in human plasma: Comparison of levels in patients with essential hypertension and normotensive control subjects. *Clin Sci* 78:261–264, 1990
184. YAMADA K, GOTO A, HUI C, SUGIMOTO T: Endogenous digitalis-like factor as a stimulator of endothelin secretion from endothelial cells. *Biochem Biophys Res Commun* 172:178–183, 1990
185. GOLIGORSKY MS, IIJIMA K, MORGAN M, YANAGISAWA M, MASAKI T, LIN L, NASJLETTI A, KASKEL F, FRAZER M, BADR KF: Role of endothelin in the development of Dahl hypertension. *J Cardiovasc Pharmacol* 17(S7):S484–S491, 1991
186. GOTO A, YAMADA K, ISHII M, YOSHIOKA M, ISHIGURO T, SUGIMOTO T: The effects of urinary digitalislike factor on cultured vascular smooth muscle cells. *Hypertension* 11:645–650, 1988
187. BOVA S, BLAUSTEIN MP, LUDENS JH, HARRIS DW, DUCHARME DW, HAMLYN JH: Effects of an endogenous ouabainlike compound on heart and aorta. *Hypertension* 17:944–950, 1991
188. FUJITA T, ITO Y: Salt loads attenuate potassium-induced vasodilatation of forearm vasculature in humans. *Hypertension* 21:772–778, 1993
189. OVERBECK HW, DERIFIELD RS, PAMNANI MB, SOZEN T: Attenuated vasodilator responses to K^+ in essential hypertensive men. *J Clin Invest* 53:678–686, 1974
190. PHILLIPS RJW, ROBINSON BF: The dilator response to K^+ is reduced in the forearm resistance vessels of men with primary hypertension. *Clin Sci* 66:237–239, 1984
191. MIZUKOSHI H, MICKELAKIS AM: Evidence for the existence of sensitizing factor to pressor agents in plasma of hypertensive patients. *J Clin Endocrinol Metab* 34:1016–1024, 1972
192. CAPPUCCIO FP, SAGNELLA GA, LETHARD LA, MARKANDU ND, MACGREGOR GA: Evidence using human arterial tissue for a circulating vascular sensitizing agent in essential hypertension. *J Clin Endocrinol Metab* 63:463–467, 1986
193. PILLAI G, SUTTER MC: Effect of plasma from hypertensive patients on contractile response of vascular smooth muscle from normotensive rat. *Can J Physiol Pharmacol* 67:1272–1277, 1989
194. TATUM HJ, MULE JG: The hypertensive action of blood from patients with pre-eclampsia. *Am J Obstet Gynecol* 83:1028–1035, 1962
195. MOROMIZATO H, MIYAGI H, NAKAYAMA M, HIGUCHI M, SAKANASHI M: Enhancement of vascular contractility by plasma substances obtained from pregnancy-induced hypertension. *Gen Pharmac* 23: 285–289, 1992
196. LABELLA FS, BIHLER I, KIM R-S: Progesterone derivatives that bind to the digitalis receptor: Effects on ^{86}Rb uptake and contractility in the isolated guinea pig heart. *Can J Pharmacol* 62:1057–1064, 1984
197. WOOLFSON RG, GRAVES J, LABELLA FS, TEMPLETON JF, POSTON L: Effect of bufalin and pregananes on vasoreactivity of human resistance arteries. *Biochem Biophys Res Commun* 186:1–7, 1992
198. ELIADES D, SWINDALL B, JOHNSTON J, PAMNANI M, HADDY FJ: Hemodynamic effects of bufalin in the anesthetized dog. *Hypertension* 13:690–695, 1989
199. PAMNANI MB, CHEN S, BRYANT HJ, SCHOOLEY JF, ELIADES DC, YUAN CM, HADDY FJ: Effects of three sodium-potassium adenosine triphosphatase inhibitors. *Hypertension* 18:316–324, 1991